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Original scientific paper

DETERMINATION OF BROCCOLI LEAF AREA AND STOMATAL NUMBER USING DIFFERENT APPLICATION METHODS OF MICROBIOLOGICAL FERTILIZER - SLAVOL

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Najdenovska Olga, Snezana Djordjevic, Elizabeta Miskoska-Milevska, Daniela Dimovska and Zoran Popovski (2013): *Determination of broccoli leaf area and stomatal number using different application methods of microbiological fertilizer - Slavol.* – Zemljište i biljka, Vol.62, No.1, 1-13, Beograd.

Application of microbiological fertilizers in agriculture has positive effect on plant production and also solves problems regarding the environmental protection. The fertilizer research is currently focusing on the effects of microbiological fertilizers on living organisms and soil. The aim of the study was to investigate the effect of different methods of application of microbiological fertilizer – Slavol on leaf area and stomatal number in broccoli "Verdia F_1" (*Brassica oleracea* L. var. *italica* Plenck.) plants. Two different methods of fertilizer application were used in this study: foliar

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application and application through irrigation water (drip irrigation system). The experiment was carried out on alluvial soil in the private farm located in Jurumleri, near to Skopje, Macedonia. Obtained results indicated that maximum average leaf area was 361.39 cm^2 in plants with foliar application of Slavol and minimum average leaf area was 167.86 cm^2 in control plants. The leaf area of plants treated with Slavol through drip irrigation system was 262.68 cm^2. It can be concluded that method of foliar application of Slavol obtained the highest values of leaf area. According to the LSD-test these values were significantly different ($p<0.01$).

Key words: broccoli, leaf area, microbiological fertilizer, stomatal number

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica* Plenck.) belongs to the genus *Brassica* and it is one of the major crop plants in this genus. It is an Italian vegetable, native to the Mediterranean region, cultivated in Italy in ancient roman times. In USA, the broccoli first appeared in 1806, but it commercially cultivation was started around 1923 (DECOTEAU, 2000). The broccoli is an important vegetable crop and has high nutritional and good commercial value (YOLDAS *et al.*, 2008).

Fertilizers are compounds usually given to plants with the intention to increase the yield and quality of crops. Over the last few decades there has been an increase in the use of organic fertilizers due to the rise in popularity of organic food production. The development of microbiological fertilizer (biofertilizer) could be connected with green organic food safety and environment protection which display a vital role in the sustainable development of high-yield, high-quality, high-efficiency agriculture (MENG *et al.*, 2008). The use of chemical fertilizers in agriculture led to deterioration of soil conditions and environment. Biofertilizers are alternative fertilizer and one of the important components of organic farming that enhances the plant growth and yield. Namely, biofertilizer is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (VESSEY, 2003). Application of biofertilizers can be expected to reduce the use of chemical fertilizers and pesticides. From the economic and ecological aspects microbiological fertilizers are much more acceptable than the use of mineral fertilizers and chemicals (MILIĆ *et al.*, 2003). Microbiological fertilizers may contain one type of microorganism or a mixture of different microorganisms. Natural bio-organic fertilizer – Slavol is made up of the combined nitrogen fixing and phosphorus mineralizing bacteria (*Azotobacter chroococcum, A. vinelandi, Derxia sp., Bacillus megaterium, B. lichenformis and B. subtilis*).

For many years scientists have investigated the positive effects of microorganisms' activities on plant production (STOLILJKOVIĆ, 1988; GOVEDARICA *et al.*, 1999; NAJDENOVSKA *et al.*, 2002a; NAJDENOVSKA *et al.*, 2002b; NAJDENOVSKA *et al.*, 2002c; NAJDENOVSKA *et al.*, 2004; NAJDENOVSKA, 2011; NAJDENOVSKA, 2012; ĐORĐEVIĆ *et al*,. 2005a; ĐORĐEVIĆ *et al.*, 2005b; BALEŠEVIĆ-TUBIĆ *et al.*, 2011). A number of plant growth-promoting rhizobacteria (PGPR) belonging to various taxonomic groups contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of the plant hormone ethylene (GLICK *et al.*, 1995 and BELIMOV *et al.*, 2005). Lowers the levels of ethylene produced in developing or stressed plants promoting root elongation. A large array of heavy metaltolerant bacteria including species of *Pseudomonas, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter Burkholderia, Bacillus and Serratia* have reported to enhance plant growth (KLOPPER *et al.*, 1989 and GLICK, 1995). Several bacteria improve plant growth through suppression of pathogens by competing for nutrients, by antibiosis, or by synthesizing siderophores, which can solubilize and chelate iron from the soil and inhibit the growth of phytopathogenic microorganisms (CABALLERO-MELLADO *et al.*, 2007).

Also, the productivity and health of horticultural crops depends on the ability of the plant cover to intercept light energy. This ability is a function of the amount of leaf area, the architecture of the vegetation cover, and plants' ability to convert light energy. Leaves are complex plant organs upon which life depends. Their primary function is process of photosynthesis. Through the process of photosynthesis they are able to trap energy in the form of sugar molecules. In order to maximize the photosynthetic production, plants increase the photosynthesizing surface. Estimation of leaf area is valuable in studies of plant nutrition, plant competition, plant-soil-water relations, plant protection measures, respiration, light reflectance and heat transfer in plants (MOHSENIN, 1986). Also, leaf area is an important parameter in understanding photosynthesis, light interception, water and nutrient use and crop growth and yield potential (SMART, 1974; WILLIAMS, 1987). Leaf area can be measured by nondestructive or destructive measurements. Agronomists require rapid, inexpensive, reliable and nondestructive method for measuring leaf area. Nondestructive estimation of plant leaf area offers inexpensive and reliable alternatives in horticultural experiments.

Stomata control the movement of gases in and out of a leaf, making carbon dioxide available for photosynthesis and controlling the loss of water from the leaf through transpiration. The number of stomata is affected by ecological condition and physiological process (DÜZENLI and ERGENOĞLU, 1991). Individual plants can change the stomatal number to adjust for changes in the environment. According to RYUGO (1988) stomatal frequency differs greatly from species to species, ranging from 125 to over 1000 stomata/mm^2. In some resources, it has been known that stomatal density changes with characteristics such as drought, net photosynthesis production (BIERHUIZEN *et al.*, 1984), precipitation change (MISIRLI and AKSOY, 1994), vegetative developmental phases and grafts on different rootstocks (ÇAGLAR and TEKIN, 1999) and altitude (ÇAĞLAR *et al.*, 2004; ASLANTAŞ and KARAKURT, 2009).

No information for effect of microbiological fertilizer – Slavol on broccoli leaf area and stomatal number can be found in the literature. The aim of the present experiment was to study the effect of different methods of application of microbiological fertilizer – Slavol on broccoli leaf area and stomatal number.

MATERIALS AND METHOD OF WORK

The study was carried out in the private farm located in Jurumleri near to Skopje, Macedonia. Broccoli "Verdia F$_1$" (*Brassica oleracea* L. var. *italica* Plenck.) plants were used as plant material. The influence of different methods of application of microbiological fertilizer – Slavol on broccoli leaf area and stomatal number was investigated. Two different application methods of fertilizer were used in this study: foliar application and application through irrigation water (drip irrigation system).

The seedlings were grown in a greenhouse. Firstly, the seedlings (about 45-days old) were treated with Slavol (10 mL/L) for few minutes, than they were transplanted in rows. The broccoli plants were planted on alluvial soil and they were treated with Slavol using different application methods during the vegetation period, starting from 21th of August until 1th of October 2012. One group of broccoli plants was treated with Slavol through the leaf (foliary) in the dose of 10 mL/L in intervals of 7 days. The second group of broccoli plants was treated with Slavol through drip irrigation system (1mL/L) in intervals of 2 days. The flow capacity of drip irrigation system was 2L /min/m^2.

Leaf area was determinate by using 30 leaf samples taken from broccoli plants one month after last application of Slavol. In same time, leaf samples from not treated (control) plants were analyzed as a control for comparison. The leaves used in this research were randomly selected from each group of broccoli plants. After cutting, the leaves were placed in plastic bags and were transported on ice to the laboratory. The leaf width (cm) was measured from tip to tip at the widest part of the lamina and leaf length (cm) was measured from lamina tip to the point of petiole intersection along the lamina midrib.

Broccoli leaf area was determined using model of nondestructive estimation of broccoli leaf area by STOPPANI *et al.* (2003). According to these authors the broccoli leaf area estimation equation was found to be, LA (cm^2) = 0.63 (leaf length x leaf width at the widest point).

Stomata on upper and lower leaf surface were counted on the middle portion of the leaf by nail polish replicas (BREWER, 1992). The prepared slides were analyzed using light microscope by a 16x ocular and a 40x objective. The number of stomata was expressed per mm^2 of leaf area. Average stomatal number was calculated from 10 leaves per treatment using counts from three areas per leaf. Collected data were statistically processed using analysis of variance (ANOVA) and the significance was evaluated by least significant differences (LSD) at $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSION

In the present study, Slavol as liquid fertilizer was applied using two different methods: foliar application and application through irrigation water. Foliar application refers to the spraying of fertilizer solution on the foliage of growing plants. Application through irrigation water (fertigation) refers to the application of water soluble fertilizers through irrigation water. In the research was used drip irrigation system. This method saves water and fertilizer by allowing water to drip slowly to the roots of plants. The drip irrigation has some advantages and disadvantages. Namely, fertilizer and nutrient loss is minimized, water application efficiency is high, weed growth is minimized and foliage remains dry, reducing the risk of disease. On the other hand, this application method of fertilizers is expensive.

According to SARIĆ *et al.* (1989) feeding plant affects numerous physiological-biochemical processes that are important for plant growth, development and yield. Application of fertilizers in vegetable crops is important for obtaining higher yields and products which are characterized by better quality (MOLNAR, 1995). Leaf is one of the important parts of the plants because it is the one which is in charge of accomplishing the photosynthesis, the respiration and transpiration. According to VOLKENBURGH (1999) the leaf expansion depends of genetic constitution and environmental conditions.

Table 1. - Average broccoli leaf area (cm^2) after using different application methods of Slavol

	Control plants	Plants with foliar application of Slavol	Plants treated with Slavol through drip irrigation system
Average leaf area (cm^2)	167.86	361.39	262.68
Minimum	60.48	144.9	90.72
Maximum	264.6	595	468.72

In this study, broccoli leaf area was estimated with nondestructive model by STOPPANI *et al.* (2003). Using new equipment and tools such as hand scanner or laser optical apparatuses for determination of leaf area is very expensive investments for basic and simple research. Development of mathematical models and equations from linear leaf measurements has been shown to be very useful in studying plant growth and development (ELSNER *et al.*, 1988; RAJENDRAN and THAMBURAJ, 1987). Non-destructive leaf area estimation also saves time as compared with geometric reconstruction. Nondestructive leaf area or plant growth measurements are often desirable because continued use of the same plants over time can reduce variability in experiments as compared with destructive sampling (NESMITH, 1991; NESMITH, 1992; GAMIELY *et al.*, 1991). Because leaf length or width are dimension that can be easily measured in the field, green-house and pod experiments, use of this model would enable researchers to make nondestructive measurements or repeated measurements on the same leaves (FALLOVO *et al.*, 2008). According to same authors, the leaf area (length: width ration) may vary among different plant materials and it is necessary to be found good model of nondestructive leaf area that will be used in physiological study independently of the genetic materials.

Average leaf area in plants treated with Slavol was larger than in control plants (Table 1). According to the data in Table 2, the LSD-test showed that the differences among the average leaf areas in control and treated plants were statistically significant ($p<0.01$). The smallest value of average leaf area (167.86 cm^2) was detected in control plants and the biggest value (361.39 cm^2) was detected in plants with foliar application of Slavol.

Table 2.- Analysis of variance for Table 1.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	378853.2	29	13063.9	1.229365	0.247974	1.662901
Columns	561854.2	2	280927.1	26.43634	6.91E-09	3.155932
Error	616339.9	58	10626.55			
Total	1557047	89				

$LSD_{0.05}$	53.24085
$LSD_{0.01}$	70.80706

This conclusion was in agreement with FRITZ (1978) who pointed out that a repeated application of small units of foliar fertilizers stimulates plant metabolism and an increased nutrient uptake via the roots can be observed. According to YILDIRIM *et al.* (2007) one of the benefits of foliar fertilization is the increased uptake of nutrients from the soil. This notion is based on the belief that the foliar fertilization causes the plant to release more sugars and other exudates from its roots into the rhizosphere. Beneficial microbial populations in the root zone are

stimulated by the increased availability of these exudates. In turn, this enhanced biological activity increases the availability of nutrients, disease-suppressive biochemicals, vitamins and other factors beneficial to the plant (YILDIRIM *et al.*, 2007). Also, KOLOTA and OSINSKA (2001) recognized that supplementary foliar fertilization during crop growth can improve the mineral status of plants and increase the crop yield.

Also, the plants treated with Slavol through drip irrigation system had bigger average leaf area (262.68 cm^2) than in control plants (Table 1). According LSD-test difference between average leaf area of plants with foliar application of Slavol and plants treated with Slavol through drip irrigation system is significant ($p<0.01$) (Table 2). From obtained data it can be concluded that highest average values of leaf area were obtained using method of foliar application of microbiological fertilizer Slavol.

KANDIL and GAD (2009) researched effects of inorganic and organic fertilizers on growth and production of broccoli and found that using organic manure plus inorganic solution fertilizers gave a significant effect on plant growth, heads yield, chemical constituents and mineral composition of broccoli. Also, their values for broccoli leaf area were from 169 to 294 cm^2. In our research, obtained value for average broccoli leaf area in plants with foliar application of Slavol (361.39 cm^2) was bigger than values for broccoli leaf area presented by KANDIL and GAD (2009).

UZUN and KAR (2004) found significant difference between leaf area of broccoli plants and planting times. According to GRANIER and TARDIEU (1998) variation in leaf size has been attributed to differences in cell number, or cell size, or combinations of the two. It is known that cell division in plants is correlated with carbohydrate supply, and that leaves starved for photosyntate or other nutrients will develop fewer cells than those growing amid plenty (DALE, 1988; CHAPIN, 1991). Many researchers (MEDINA, 1970; GULMON and CHU, 1981) have reported reducing leaf area under limiting nitrogen. Microbiological fertilizers which contain highly efficient strains of bacteria, fungi and algae provide plants with biogenous elements: nitrogen, phosphorus and potassium (GOVEDARICA *et al.*, 2002). In previous studies was confirmed positive effect of microbiological fertilizer – Slavol on plant production (ĐORĐEVIĆ *et al.*, 2005a; JELAČIĆ, 2007; MISIMOVIĆ *et al.*, 2012).

Our data indicated that the nitrogen fixing and phosphorus mineralizing bacteria present in the microbilogical fertilizer - Slavol probably increase the supply of plant nutrients that improve the nutritional status of plants and increased leaf size in treated plants. This is in agreement with study of NAJDENOVSKA *et al.* (1998). Namely, according to these authors nitrogen fixing bacteria synthesize substances of growing so that they improve the growing and development of plants, as well as the resistance of the plants to illnesses and harmful insects.

According to JONES *et al.* (1987) stomatai dimension and especially frequency can change more than two fold in response to radiation, to water status or according to developmental stage. The leaves of broccoli are amphistomatous i.e. stomata are on upper and lower epidermis. According to ASTON (1978) stomata on upper epidermis are more sensitive to environment variables.

Table 3. - Average stomatal number of broccoli leaves (stomata/mm²) after using different application methods of Slavol

	Control plants	Plants with foliar application of Slavol	Plants treated with Slavol through drip irrigation system
Average stomatal number on upper epidermis (stomata/mm²)	145	102	140
Average stomatal number on lower epidermis (stomata/mm²)	250	232	242
Total stomatal number (stomata/mm²)	395	334	382

As presented in Table 3, stomatal number on lower epidermis is bigger than on upper epidermis. Average stomatal number on upper and lower epidermis of control plants was 145 and 250 stomata/mm², respectively. Also, in plants treated with Slavol through drip irrigation system stomatal number on upper epidermis were 140 stomata/mm² and 242 stomata/mm² on lower epidermis. On the other hand, average stomatal number on upper and lower epidermis of plants with foliar application of Slavol was 102 and 232 stomata/mm², respectively (Table 3).

No significant difference was detected between stomatal number on upper epidermis in control plants and plants treated with Slavol through drip irrigation system (Table 3). The plants with foliar application of Slavol had the lowest stomatal number on upper epidermis compared with control plants and plants treated with Slavol through drip irrigation system. According to the LSD-test these differences were statistically significant ($p<0.01$).

Also, no statistically significant difference was found among treatments and control refer to stomatal number on lower epidermis.

The difference between the total stomatal numbers in control plants and plants treated with Slavol through drip irrigation system was not statistically significant. In plants with foliar application of Slavol was measured the lowest total stomatal number (334 stomata/mm²). According to the SDS-test, the difference between the control plants and plants with foliar application of Slavol was statistically significant ($p<0.01$). Also, analyses of total stomatal number in plants treated with Slavol through drip irrigation system and plants with foliar application of Slavol showed statistically significant difference ($p<0.05$) (Table 4).

Stomatal characteristics are one of the main factors that determine photosynthetic ability and the genetic basis for stomatal frequency is still poorly understood (ARMINIAN *et al.*, 2008). According to these authors phenotyping may not have been sufficiently precise, there was a strong environmental influence or a large genotype by environment interaction was involved in variability of these traits. Also, these authors suggest that stomatal characteristics are not an appropriate approach to breed high yielding wheat varieties. KÜRSCHNER (1997) demonstrated empirically that a difference exists in stomatal density and stomatal index response during leaf expansion depending upon the position of the leaf in tree crown.

Table 4.- Analyses of variance table for Table 3 (total stomatal number)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	134667.1	29	4643.694	0.775841	0.769357	1.662901
Columns	62787.22	2	31393.61	5.245058	0.008058	3.155932
Error	347151.4	58	5985.37			
Total	544605.8	89				

$LSD_{0.05}$ 39.95714

$LSD_{0.01}$ 53.14053

From obtained results, no general conclusion can be given refer to effects of application methods of fertilizer – Slavol on stomatal number in broccoli leaf.

CONCLUSION

By this experiment it is proved that microbiological fertilizer –Slavol had positive statistically significant effect on leaf area in both group of plants where was used different method of application of microbiological fertilizer – Slavol. Also, it can be concluded that highest average values of leaf area were obtained using method of foliar application of microbiological fertilizer Slavol. The LSD-test showed that differences among plants treated with microbiological fertilizer – Slavol and control plants were statistically significant ($p<0.01$).

In plants with foliar application of Slavol were detected the lowest total stomatal number compared with control plants and plants treated with Slavol through drip irrigation system. According to the LSD-test the difference between control and plants with foliar application of Slavol was statistically significant ($p<0.01$). Also, the difference between plants treated with Slavol through drip irrigation system and plants with foliar application of Slavol was statistically significant ($p<0.05$).

The results presented here support the hypothesis that microbiological fertilizers can improve plant grow but, more studies are needed to be better understand effect of microbiological fertilizers on living organisms and soil.

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OPREDELENJE LISNE POVRŠINE I BROJ STOMA U BROKULI KORISTEČI RAZLIČITE METODE APLIKACIE MIKROBIOLOŠKOG ĐUBRIVA – SLAVOL

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Izvod

Primena mikrobioloških đubriva u poljoprivredi ima pozitivan uticaj na biljnu proizvodnju, a isto tako rešava probleme vezane za zaštitu životne sredine. Trenutna istraživanja fokusiraju se na efekte mikrobioloških đubriva na žive organizme i zemljišta. Cilj istraživanja je bio da se ispita uticaj različitih metoda aplikacije mikrobiološkog đubriva - Slavol na lisnu površinu i broj stoma u brokuli "Verdia F_1" (*Brassica oleracea* L. var. *italica* Plenck.). U istraživanju korišćene su dve različite metode aplikacije đubriva: folijarna aplikacija i aplikacija kroz vodu za navodnjavanje (sistem kap po kap). Ogled je postavljen na aluvijalan tip zemljišta na privatnom posedu u selu Jurumleri, u blizini Skoplja, Makedonija. Dobijeni rezultate pokazuju da je maksimalna prosečna površina lista bila 361.39 cm^2 u biljaka koje su bile folijarno tretirana sa Slavolom, dok minimalna prosečna površina lista bila je 167.86 cm^2 u kontroli. Lisna površina biljaka tretirane sa Slavolom kroz sistem za navodnjavanje bila je 262.68 cm^2. Prema LSD-testu, ovi podatci su pokazale statističku razliku ($p<0.01$). Može se zaključiti da metoda folijarne aplikacije Slavola daje najveće vrednosti za površinu lista.

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